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PROVISIONAL APPLICATION COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION under 37 CFR 1.53(c).

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TITLE OF THE INVENTION (280 characters max)

VIRULENT PHAGE FOR DETECTING LISTERIA MONOCYTOGENES IN FOODSTUFFS AND IN FOOD PROCESSING PLANTS

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ENCLOSED APPLICATION PARTS *(check all that apply)*

- ☒ Specification *Number of Pages* 12
- ☐ Drawing(s) *Number of Sheets* ____
- ☐ Small Entity Statement
- ☐ Other (specify)

METHOD OF PAYMENT *(check one)*

☒ Check # 342649 is enclosed to cover the Provisional filing fees ☒ The Commissioner is hereby authorized to charge any underpayment or credit any overpayment to Deposit Account No. 01-2300.	PROVISIONAL FILING FEE AMOUNT(S)	$160.00

The invention was made by an agency of the United States Government or under a contact with an agency of the United States Government.

☒No
☐Yes, the name of the U.S. Government agency and the Government contract number are:

Respectfully submitted,

SIGNATURE _____ Date: July 8, 2002
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☐Additional inventors are being named on separately numbered sheets attached hereto.

PROVISIONAL APPLICATION FILING ONLY

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A P P L I C A T I O N

for

UNITED STATES LETTERS PATENT

on

VIRULENT PHAGES TO CONTROL LISTERIA MONOCYTOGENES IN FOODSTUFFS AND IN FOOD PROCESSING PLANTS

By

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Docket No.: 108026-00012

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VIRULENT PHAGES TO CONTROL LISTERIA MONOCYTOGENES IN FOODSTUFFS AND IN FOOD PROCESSING PLANTS

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to the use of a particular class of bacteriophages ("phage") known as virulent phages that are lytic for the bacterial species Listeria monocytogenes, and which is shown in the present invention to reduce the counts of these bacteria and/or to prevent their growth in the first place, in foods products (including but not limited to the dairy industry) as well as on processing equipment and other sites in food industry facilities, and as a therapeutic agent for treating animals infected with Listeria monocytogenes. One specific example of a virulent Listeria monocytogenes phage, is a phage designated P100, recently discovered by one of the present inventors. The present invention also relates to methods that will enable additional phage that have lytic properties to be developed and/or isolated.

Phages, as antibacterial agents, have the advantage of replicating within the bacterial target. Thus, when their progeny lyse the cell and escape into the extracellular milieu, they can infect and multiply in succeeding generations of bacteria, producing progeny levels far greater than that of the binary growth of the target bacteria, thereby increasing the phage population exponentially in numbers at the expense of the bacterial targets.

The concept of using phages to identify bacterial contamination in food products {and facilities, equipment}, in general, has been described in the scientific literature (see, e.g. Greer, J. Food Prot., 49:104-109, 1986). The concept of using Listeria phages in particular, to identify Listeria contamination of dairy products and facilities/equipment in specific, was described as early as 1990 (Loessner et. al., Applied and Environmental Microbiology, June 1990, p.1912-1918). The present invention concerns the use of a

recently discovered Listeria phage with specific, essential and relevant properties, which makes it particularly suitable for identifying and controlling Listeria contamination of dairy products, facilities and equipment.

In addition to the general scientific literature on the subject, there is also patent literature that teaches the utility of phages in general to control bacterial contaminations in food processing plants and in foodstuffs. See for example U.S. Patent No. 5,006,347 issued on April 9, 1991, U.S. Patent No. 4,851,240 issued on July 25, 1989, and EP 0414304A2 published on February 27, 1991. However, none of the above discussed patents disclose a Listeria phage which was actually tested and shown to successfully control bacterial contamination in food processing plants and in food products. The reason for this is that all of the Listeria phages known in the art at the time of the disclosure in the previous patents were temperate phages, and were therefore not efficient at nor suitable for industrial bacterial eradication purposes. The term "temperate" refers to the fact when a strain of phage injects its DNA into a bacterial target, the phage DNA integrates into the DNA of the host cell, as a "prophage", and can remain integrated therein for considerable periods of time. Since the prophage excises (and initiates replication and lysis) only when the host cell becomes stressed, the ensuing bacterial lysis is unpredictable and not easily controlled, which is why temperate phages do not lend themselves well to industrial applications. Temperate phages are unsuitable for industrial decontamination purposes for other reasons as well, including the fact that they can deliver unwanted and dangerous genes to the bacteria target into which their DNA integrates. In contrast, there is a class of phages that lyse bacterial targets directly, given that they do not have the molecular machinery required to integrate into the bacterial targets. Such phages are referred to as being "virulent" or "lytic" for the bacterial targets. Virulent phages against Listeria monocytogenes were discovered recently, by one of the present inventors.

The first of these virulent Listeria phages, designated A511, was described in the literature in 1990 (see Loessner et. al., Applied and Environmental Microbiology, June 1990, p.1912-1918, 1990). The virulent phage according to the present invention belong

to the Myoviridae family and have tails which contract towards the virus head. One particularly preferred phage is designated P100 and was deposited at the American Type Culture Collection, 10801 University Blvd., Manassas VA 20110-2209 on ----, 2002, accession number PTA-4383.

The virulent phages described in the present invention can also be used against CFUs (colony forming units) of Listeria monocytogenes bacteria that are in biofilms, as opposed to CFUs that are planktonic. The use of temperate Listeria monocytogenes phages against Listeria biofilms has been described in the literature (see e.g. Roy et. al., Appl. Environ. Microbiol., Sept., 59(9):2914-7, 1993). Specifically, Roy et. al. used temperate Listeria bacteriophages H387, H387-A, and 2671 of the Siphoviridae family. While these temperate phages demonstrated some efficacy in clearing a Listeria biofilm, even when used in combination the best they could obtain was a 3.5 – 3.7 log reduction in counts. Roy et al indicates that such reductions "will have to be improved on to meet the recommended reduction level of 99.999% in a 30-s exposure for a chemical sanitizing agent". As stated above, temperate phages are not predictable or readily controllable in the timing of or efficiency with which they can kill the target bacteria.

In addition to the use of the virulent phages described above, the present inventors have also found that virulent substrains can be derived from a number of temperate phage strains. These virulent substrains can be selected by techniques such as plaque isolation (in which one selects the clearest areas of a plaque, and enriches for the most virulent strains therein by repeated cycles of growing to high titer, plating for new plaques, and picking the clearest areas of the later-generation plaques). Examples of temperate phage strains, from which virulent substrains have been or will be developed, include the temperate strains designated A118, A502, A006, A500, PSA, P35, and related viruses.

2. Description of the Related Art

Listeria monocytogenes is a bacterial pathogen that contaminates many food products, the list of which includes but is not limited to soft cheeses, patés, ice cream, smoked and cured fish, frozen seafood, salads, and processed meats. When ingested, these bacteria can produce a disease termed listeriosis, characterized by a variety of symptoms and conditions, including diarrhea, abortion, and encephalitis. Collectively, in the industrialized nations, hundreds of deaths occur each year as a result of Listeria monocytogenes food contamination.

The food processing industry has not been sufficiently successful in eradicating Listeria monocytogenes bacteria from the environment of the processing plants. As a result, even foods that have been pasteurized at temperatures high enough to kill these bacteria nevertheless become contaminated, post-pasteurization. The bacteria gain access to the foodstuffs through one or more routes, including (i) from the raw materials (e.g. raw milk, and/or milk that has been pasteurized at low temperatures); (ii) from the processing machinery (in and on which the bacteria can grow as biofilms that are difficult to eradicate); and (iii) from airborne bacteria present in the plant environment which can settle onto the surface of the foodstuffs during curing, packaging, and so on.

Despite the numerous methods used in the food industry to control and prevent L. monocytogenes contamination, the bacteria gain access to and persist in the environment of food processing plants. Moreover, they survive the very high concentrations of salt that are present in several food-making processes. The resulting contamination of the foodstuffs (including but not limited to cheeses, patés, cold cuts, hot dogs and other processed foods) leads to scores of deaths each year in developed nations, and also to product recalls whose retail worth each year, in the aggregate, is measured in the hundreds of millions of dollars.

The methods currently in use to control <u>Listeria</u> in the food industry include: (i) pasteurization of primary ingredients (e.g. milk) and heat treatment of the products, which is often unsuccessful because recontamination frequently occurs and many

products cannot undergo a final (listeriocidal) heat treatment; (ii) application of physicochemical agents such as disinfectants, enzymes, antibiotics, etc., which experience has shown do not reduce the bacterial counts sufficiently; and (iii) attempts to break up biofilms mechanically, which leave sufficient residues of bacteria behind that the foodstuffs still become contaminated.

Additional methods must therefore be made available to the food processing industry in order to protect the health of consumers, and to reduce the exposure of numerous companies to the great cost and the loss of good will that result from such contaminations and recalls.

The present inventors have conducted a series of experiments using a strain of L. monocytogenes that is prevalent in the processing plant of a particular manufacturer of soft ("spread") cheeses. That bacterial strain proved to be susceptible to phage P100 in vitro. As will be shown in the Examples section, phage P100 proved able to reduce below measurable/detectable limits the Listeria bacteria that had been spiked into a cheese-like matrix.

SUMMARY OF THE INVENTION

Virulent phage P100, as well as other virulent phages from the Myoviridae and Siphoviridae families, and virulent mutants of various temperate strains of phage (such as but not limited to phages B054, A118, A502, A006, A500, PSA, P35 and related viruses) are used in the present invention to control Listeria monocytogenes bacteria present on (or within) foodstuffs, as well as those Listeria monocytogenes bacteria present in the equipment or the general environment of the food processing plants in which the foodstuffs are being processed. These phage can also be used to treat animals infected with Listeria monocytogenes.

The present invention is directed to the use of a class of Listeria monocytogenes phages which are particularly suitable for bacterial control methods. The phage are preferably from the Myoviridae family and are virulent against Listeria monocytogenes

[redacted]

strains of serovar 1/2. In addition, the present invention isolates virulent mutants of temperate strains and uses those specific mutants in the control of bacterial contamination of foodstuffs and of food processing plants.

DESCRIPTION OF PREFERRED EMBODIMENTS

The above-referenced phages are applied on or into food products, and/or into various physical sites within the food processing plants, by a number of means including, but not limited to, admixing the phages into the food products, spraying them onto the foodstuffs, spraying them onto the plant equipment, and/or directly applying them to the plant equipment. Said phage applications significantly reduce the numbers of Listeria monocytogenes bacteria that would otherwise be present.

The phage of the present invention can also be used to treat animals, including humans, infected with Listeria monocytogenes. Any suitable route of administration can be used to administer the phage including but not limited to: oral, aerosol or other device for delivery to the lungs, nasal spray, intravenous, intramuscular, intraperitoneal, intrathecal, vaginal, rectal, topical, lumbar puncture, intrathecal, and direct application to the brain and/or meninges. Excipients which can be used as a vehicle for the delivery of the phage will be apparent to those skilled in the art. For example, the free phage could be in lyophilized form and be dissolved just prior to administration by IV injection. The dosage of administration is contemplated to be in the range of about 10^3 to about 10^{13} pfu/per kg/per day, and preferably about 10^{12} pfu/per kg/per day. The phage are administered until successful elimination of the Listeria monocytogenes is achieved or until the amount of Listeria monocytogenes is substantially reduced.

The present invention also covers the use of the phages, when used in combination with other anti-Listerial agents known in the art. Examples of such anti-Listerial agents, which are preferentially combined with phages, include but are not limited to:

1. Endolysins (phage lysins):

The phage of the present invention can be combined with listeriolysins which are enzymes which have been shown to selectively control Listeria in food and the environment (DE4326617C1 and EP 95932002.9)

2. Surface disinfectants:

The phages of the present invention can be combined with known surface disinfectants such as (i) preservatives of various kinds, such as but not limited to benzoic acid and BHT; and (ii) various disinfectants with which the phages are compatible, such as but not limited to quaternary ammonium compounds.

3. Antibiotics

The phage of the present invention can be used in combination with known antimicrobial agents (including antibiotics and chemotherapeutic agents) including but not limited to vancomycin, nisin, danofloxacin and neomycin.

4. Enzymes

The phage of the present invention can be used in combination with enzymes to aid in breaking up biofilms (e.g. biofilms found in food processing equipment). Such enzymes are known in the art and include but are not limited to polysaccharide depolymerase enzymes, and protease.

5. Surfactants

The phage of the present invention can be combined with known surfactants when used to treat food processing equipment. The surfactant helps to wet the surface so that the phage are properly distributed over the various surfaces, and to solubilize and remove dirt so that the Listeria are accessible to the phage. Suitable surfactants include but are not limited to Tween 80, 20 and 81 and Dobanols.

6. Bacteriophages specific for bacterial contaminants other than Listeria monocytogenes

The phage of the present invention can be combined with phage specific for other bacteria known to contaminate food processing equipment and food products. Such bacteria include but are not limited to E. coli, and bacterial species from the genera Salmonella, Bacillus, Staphylococcus, Streptococcus, Clostridium, and Pseudomonas.

The phage can be applied in a liquid or a powdered form to food products and food processing equipment. If applied as a liquid, the phage are applied at a concentration of 10^3 to 10^{10} PFU (plaque forming units) per mL and preferably at a concentration of 10^6 to 10^9 PFU (plaque forming units) per mL. If applied as a dry powder the phage are applied at a concentration of 10^3 to 10^{10} PFU (plaque forming units) per mg and preferably at a concentration of 10^6 to 10^9 PFU (plaque forming units) per mg. The phage can be suspended in a suitable carrier prior to application or drying, including but not limited to protein solutions containing BSA, casein, whey protein, soy bean protein, etc and sugar based carriers containing sugars such as mannitol. The phage can be lyophilized or cryopreserved by vitrification and either suspended in a solution prior to application or applied directly as a dry powder.

Suitable amounts of phage for use in the present invention can be obtained by techniques known in the art, including but not limited to a batch technique where a culture of host bacteria is grown and then seeded with phage. After an amount of time suitable to allow maximal phage propagation and bacterial lysis, the culture is further lysed by physical or chemical means and the lysate spun down. The phage containing supernatant can be used as is or further purified using techniques such as ultrafiltration, chromatography and centrifugation.

As used in the present application, the term "dairy product" is intended to include any food product made using milk or milk products, including but not limited to milk, yogurt, ice cream, cheese, butter, and cream.

As used in the present application, the term "meat product" is intended to include any food product which contains animal tissue, including but not limited to beef, pork,

and poultry. The term "ready to eat meat product" in intended to include any meat product which does not require cooking prior to consumption., including but not limited to patés, hot dogs, bologna, salami, and cold cuts.

As used in the present application, the term "fish product" is intended to include any food product which contains tissue from an aquatic animal including but not limited to lobster, crab, fresh water and saltwater fish and other seafoods.

As used in the present application, the term "unpasteurized food product" is intended to include any food product which is prepared using unpasteurized primary ingredients and which does not undergo a final (listeriocidal) heat treatment.

As used in the present invention, the term "salad" is intended to include any food product which contains mixtures of vegetables or fruits, and particularly such mixtures as are presented for consumers to choose from in a display commonly referred to as a "salad bar".

EXAMPLE

Experiment 1:

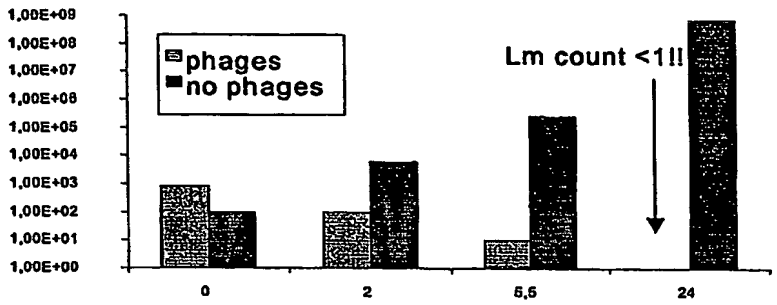
Step 1. 10E3 CFUs of Listeria monocytogenes are placed in a liquid culture.

Step 2. 5x10E8 PFU of phage P100 are mixed into the liquid culture.

Step 3. As a control, the buffer in which phage P100 was suspended was mixed into an aliquot of the liquid culture.

Step 4. Colony counts of the bacteria were performed at various intervals of time.

Results:



With Listeria monocytogenes inoculated at 10^3 CFU per mL of liquid culture, and phages added at a concentration of $5x10^8$ PFU per mL → virtually complete eradication of Listeria bacteria was achieved

ABSTRACT OF THE DISCLOSURE

The present invention relates to virulent (lytic) Listeria monocytogenes phage from the Myoviridae family, preferably P100, alone or in combination with other virulent phages. P100 can be administered to food products, to the components that will be added to food products, and/or to the infrastructure of the food processing plants within which such food products are processed, or the containers or wraps in which such foods are stored and/or shipped, in order to reduce Listeria monocytogenes contamination. The phage of the present invention can also be used to treat animals infected with Listeria monocytogenes. P100 will kill the bacteria that are within its host range with great efficiency and will propagate to high titer thereon. P100 can be combined with other lytic phage, and/or with other antimicrobial agents to reduce or eliminate Listeria.